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Genetic diversity among Chinese and Egyptian garlic (*Allium sativum* L.) germplasm accessions based on 19 morphological traits and 16 new microsatellites marker

Hassan H.A. Mostafa[1,2], Wang Haiping[1], Song Jiangping[1], Zhang Xiaohui[1] and Li Xixiang[1]

[1]Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences; The Key Laboratory of Biology and Genetics Improvement of Horticultural Crops, Ministry of Agriculture; 12 Zhongguancun, Nandajie, Haidian District, 100081Beijing, China.
[2]Central Laboratory of Organic Agriculture, Agricultural Research Centre (Affiliation ID: 60019332), 9 Gamaa Street, 12619 Giza, Egypt.

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ABSTRACT
This investigation aims to develop new SSR primers for assessing the genetic variation of garlic (*Allium sativum*, L.) germplasm resources collected from Egypt and China, and also between selected clones and their parents, to provide useful information for efficient management and enhancement of garlic germplasm resources. Genetic diversity of Chinese (83 accessions) and Egyptians (21 accessions) garlic germplasm were analyzed using 19 morphological traits and 16 newly developed microsatellites (SSR) primers. The 16 new SSR primer pairs generated a total of 45 alleles across the 104 garlic germplasm. The number of alleles revealed by each primer pair ranged from 2 to 4 and the average polymorphic alleles per locus was 2.81. The allele frequency ranged from 0.4183 to 0.9183. The gene diversity ranged from 0.1529 to 0.6670. Moreover, the observed PIC (Polymorphism Information Content) ranged from 0.1463 to 0.6175 and the average of heterozygosity was 0.4945. The dendrogram constructed based on the SSR data divided the 104 garlic germplasm into three main clusters. The clustering analysis based on morphological characters separated all the germplasm into two major clusters. In the two dendrograms, the genetic relationships among accessions are basically similar. Generally, it could be noticed that both of dendrograms were almost in accordance with geographical origin. Besides, the analysis result by 12 primers of 16 novel SSR primers confirmed the genetic variation between selected clones and their parents, which shows that clonal selection from a natural variation population can be effective for genetic improvement in garlic.

***Keywords*:** Clonal selection, cluster analysis, geographical location, novel SSR

Introduction

Garlic (*Allium sativum* L.) with high economical values has been grown all over the world from temperate to subtropical climates (Fritsch and Friesen, 2002). According to FAO statistics, China is the leading producer of garlic in the world, with the production accounting for 77.4% of the world (FAO, 2012). China has vast land geologically where climatic conditions vary greatly. The garlic germplasm in China consists of numerous famous local varieties evolved as a result of long-term natural and artificial selections under various ecological environments. Additionally, Egypt is also one of the major producers of garlic with the highest average yield of 25.287 ton/ha^{-1} recorded (FAO, 2012). There are also many local varieties of garlic there.

Despite the importance of garlic and abundance of germplasm resources, very limited breeding work has been done in garlic so far (Agrawal *et al.*, 2003). The main breeding method for garlic is clonal selection from natural population, since the plant sterility precludes its improvement through cross-hybridization (Lampasona *et al.*, 2003; Jo *et al.*, 2012). As the first step of systemic breeding program, estimation of genetic diversity is important as a prerequisite for crop genetic improvement (Agrawal *et al.*, 2003). Furthermore, garlic germplasm collection and its genetic estimation will be useful to obtain elite cultivars for clonal selection (Figliuolo *et al.*, 2001). The traditional method of morphological characterization is common in plant breeding although it has some drawbacks, such as

Corresponding Author: Li Xixiang, Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, The Key Laboratory of Biology and Genetics Improvement of Horticultural Crops, Ministry of Agriculture; 12 Zhongguancun, Nandajie, Haidian District, 100081Beijing, China.E-mail: lixixiang@caas.cn

it is descriptive, error-prone, and affected by the environmental or physiological factors. Molecular characterization is not affected by environmental factors (Jo *et al.*, 2012). The advantage of molecular methods is their capacity to detect genetic diversity at a higher level or resolution than other methods. Therefore, molecular method is supporting the classic methods, such as morphological and physiological traits (Latif *et al.*, 2013). Hence, morphological traits along with molecular markers have great importance for breeders to assess genetic diversity of germplasm because it is more reliable and consistent (Vijayanand *et al.*, 2009). Microsatellites (simple sequence repeats, SSR) are considered to be the efficient markers for genetic diversity studies in many plants. Because of their polymorphism, reproducibility and co-dominant nature, they are still the markers for compilation, standardization and exchange of information concerning genetic resources (Rakoczy-Trojanowska and Bolibok, 2004; Karuri *et al.*, 2010; Jo *et al.*, 2012; Moncrief *et al.*, 2016). Furthermore, SSR markers are used in linkage map construction and marker assisted selection of certain important characters through identification of the molecular markers for crop improvement (Pandian *et al.*, 2018). Moreover, Singh *el al.* (2018) reported that multiallelic SSR markers are more efficient than SNP markers for genetic diversity analysis because resolving power for clustering increases with the number of alleles per locus.

Currently, SSR markers are widely used for genetic diversity analysis, phylogenetic and population genetic studies (Pandian *et al.*, 2018). Many investigations have been conducted of the genetic diversity of garlic germplasm using phenotype traits and SSR markers. However, only two studies (Lee *et al.*, 2011; Cunha *et al.*, 2012) have been involved in developing new SSR primers to investigate the genetic diversity of garlic germplasm. Furthermore, little information regarding the genetic diversity of Egyptian garlic germplasm is available. Therefore, more representative germplasm from the area of its origin and more primers are needed for more comprehensively and accurate understanding of the genetic diversity of garlic germplasm. Moreover, genetic diversity analysis is necessary for productivity improving of garlic through elite germplasm selection for direct commercial use or in breeding programs.

The objectives of the present investigation are to develop more new SSR primers, to understand the genetic diversity of garlic germplasm resources from Egypt and China, and to provide useful information for the collection and conservation of garlic genetic resources and for garlic breeding.

Materials and Methods

1. Plant materials

A total of 104 garlic germplasms were evaluated, including 83 garlic germplasms from 19 provinces in China provided by the national germplasm repository for vegetatively propagated vegetables, Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, China and 21 garlic germplasm collected from four geographical areas in Egypt. All accessions used in the present study are listed in Table 1. For each germplasm, five individuals were randomly chosen for morphological characterization and their mixture is used for molecular evaluation.

2. Morphological characterization

All garlic germplasm from China and Egypt were planted at Beijing Research Station of Vegetable Crop Gene Resource and Germplasm Enhancement, Langfang, Hebei, China under the same ecological and agronomic conditions, with one plot (400 cm x 90 cm) per germplasm, at a density of three rows per plot with 10 cm between plants. Quantitative and qualitative traits (19 traits), were used to measure morphological characteristics based on International Plant Genetic Resources Institute (IPGRI) descriptor (2001) for *Allium* species (Table 2).

3. SSR primer development

Transcriptome profiles were established from two Chinese garlic germplasm (8N017 and 8N036) (data have not been published). SSR motifs were screened and primer pairs were designed online by using batchprimer3 software (Frank *et al.* 2008) at http://batchprimer3.bioinformatics.ucdavis.edu/cgi-bin/batchprimer3/batchprimer3.cgi, with manual adjustments. The mononucleotide repeats were excluded and the microsatellite loci only contain di-nucleotide ≥6, and from tri-nucleotide to hexa-nucleotide motifs ≥ 5 repeats. Primers length were ranged from 18 to 25 with optimal length 20 nucleotides, annealing temperature (Tm) from 55 to 60 °C (optimum Tm was 58 °C), and the optimal

primer GC content was 50% to obtain product size ranging from 80 to 150 bp with optimal 100 bp.

Table 1: The code and origin of garlic germplasm from China and Egypt used in the study.

Germplasm code	Origin	Germplasm code	Origin	Germplasm code	Origin
8N002A	China-Sichuan	8N141C	China-Shandong	8N260	China-JiangSu
8N002B	China-Sichuan	8N141*	China- Shandong	8N261	China-JiangSu
8N004	China-Sichuan	141°	China- Shandong	261°	China-JiangSu
8N016	China-Sichuan	8N145	China-SiChuan	261*	China-JiangSu
8N017	China-Sichuan	8N149	InnerMongolia	8N263	China-JiangSu
8N017*	China-Sichuan	8N155	China- Shandong	8N264	China- Shandong
8N024	China-Hubei	8N156	China- Shandong	8N273	China-JiangSu
8N026B	China-Hubei	8N172	China-Yunnan	8N274	China-JiangSu
8N027	China-Jiangxi	8N173	China-HeiLongjiang	8N275	China-JiangSu
8N028	China-Jiangxi	8N180	China-Hebei	8N306	China-Yunnan
8N032	China-Shanxi	8N188	China-Guizhou	8N321	China-Yunnan
8N034	China-Beijing	8N197	China-Gansu	8N326	China-Yunnan
8N035	China- Shandong	8N200	China-He´nan	8N362	China-ZheJiang
8N036	China- Shandong	8N202	China-Ningxia	8N494	Egypt-ElMinia
8N036-1*	China- Shandong	8N205	China-Gansu	8N495	Egypt-ElMinia
8N36-2*	China- Shandong	8N206A	China-Hebei	8N496	Egypt-ElMinia
8N043	China- Shandong	8N206B	China-Hebei	8N497	Egypt-ElMinia
8N045	China- Shandong	8N207	China-Hebei	8N498	Egypt-ElMinia
8N047	China- Shandong	8N209	China-Liaoning	8N499	Egypt-ElMinia
8N060	China-Shanxi	8N212	China-JiangSu	8N500	Egypt-ElMinia
8N066A	China-Shanxi	8N218	China-JiangSu	8N501	Egypt-ElMinia
8N066B	China-Shanxi	8N219	China- Shandong	8N510	Egypt-ELMinia
8N069	China-Shanxi	8N232	China-Hubei	8N511	Egypt-Assiut
8N078A	China-Shanxi	8N236	China-Hebei	8N512	Egypt-Assiut
8N099	China-Tibet	8N238	China-JiangSu	8N513	Egypt-El Mansoura
8N102A	China-HeiLongJiang	8N239	China-JiangSu	8N514	Egypt-Qena
8N113	China - Hubei	8N241	China-Yunnan	8N515	Egypt-Qena
8N118	China - Shaanxi	8N242	China-Yunnan	8N516	Egypt-Assiut
8N120	China - Shaanxi	8N245	China-Yunnan	8N517	Egypt-Assiut
8N122	China - JiangSu	8N248	China-Yunnan	8N518	Egypt-Assiut
8N124	China - JiangSu	8N254A	China-Yunnan	8N519	Egypt-Assiut
8N128	China - JiangSu	8N254B	China-Yunnan	8N520	Egypt-Assiut
8N129	China - JiangSu	8N257	China-JiangSu	8N521	Egypt-ElMinia
8N141A	China- Shandong	8N258	China-JiangSu	8N522	Egypt-ElMinia
8N141B	China- Shandong	258*	China-JiangSu		

*Clones obtained using single plant selection
°Clones gotten using bulk selection

Table 2: Morphological characters and their units/grading based on IPGRI descriptor (2001) for *Allium* species.

No.	Quantitative traits	Unit	No.	Qualitative traits	Unit
1	Plant height	cm	1	Plant type	1: Erect 2: Semi-erect 3: Spreading
2	Plant breadth	cm	2	Rigidity of leaf	1: Drooping 2: Semi-drooping 3: Erect
3	Leaf length	cm	3	Leaf transverse section	1: Flat 2: "V" shape 3: Cannular
4	Leaf width	cm	4	Leaf color	1: Yellowish green 2: Light green 3: Green 4: Dark green
5	Number of leaves per plant	number	5	Leaf wax	1: Absent 2: Little 3: Medium 4: Much
6	Overground pseudostem height	cm	6	Sheath color	1: White 2: Greenish white 3: Red 4: Purplish red
7	Overground pseudostem diameter	cm	7	Pseudostem transverse section	1: Round 2: Elliptic
8	Bulb weight	g	8	Bolting	1: No bolting 2: Incomplete bolting 3: Bolting
9	Bulb length	cm			
10	Bulb diameter	cm			
11	Number of cloves per bulb	number			

4. DNA extraction

Young healthy leaves were collected from five plants for each garlic germplasm as a bulk for

genomic DNA extraction. Samples were placed in freezer at -80 °C over night, and then dried with a freeze dryer (Beijing Boyikang Laboratory Instruments co., Ltd) and ground into fine powder. Approximately, 100 mg of garlic powder was put into a 2 ml micro-centrifuge tube to which the total genomic DNA was then extracted using the Cetyl Trimethyl Ammonium Bromide (CTAB) method as previously described (Murray and Thompson 1980). The quality and quantity of DNA were assessed by measuring absorbance at 260/280 nm and 260/230 nm and path length 0.7mm with BioSpec-nano (SHIMADZU Biotech) and the concentrations of DNA were adjusted to 20ng μl^{-1} before conducting PCR.

5. DNA fragment amplification

Amplification of our targeted DNA fragment was done using polymerase chain reaction (PCR) which was performed in a final volume 20 µl containing 2 µl of DNA template (20 ng/1µl), 2 µl of 1 x PCR buffer (containing Mg^{2+}), 1.6 µl dNTP (2.5mM each), 0.6 µl of 5 µM forward primer, 0.6 µl of 5 µM reverse primer, 0.3 µl of *Tag* polymerase enzyme (2.5 U/µl) and 12.9 µl ddH_2O. The PCR condition was as follows: denaturation step at 94 °C for 3 min, followed by 35 amplification cycles of 30 sec at 94 °C, 45 sec at 55 °C, 1 min at 72 °C; and a final extension step at 72 °C for 10 min. The amplification products were separated on 8 % denatured polyacrylamide (acrylamide:bisacrylamide, 19:1) gel. The electrophoresis was performed in 0.5 % of 10 x TBE buffer and the running time was 1.5 h at 160 volts. Gels were stained using silver nitrate ($AgNO_3$) solution (0.2%) for 12 minutes. The size of each band was approximately estimated by 50 bp DNA ladder (50 – 500 bp) (Tiangen Biotech Co., LTD., Beijing, China).

6. Data analysis

MS-Excel was used for data summarization and calculation. Cluster analysis of the morphological traits of all germplasms in this study was performed using the un-weighted pair group method with arithmetic averages (UPGMA). Regarding to the SSR data, a binary data matrix indicating the presence (1) or the absence (0) of bands (Lee *et al.*, 2011) was scored manually from SSR profiles to perform a cluster analysis. A dendrogram presenting the genetic relationships among germplasms based on simple matches coefficient (Sokal and Michener, 1958) by using the un-weighted pair group method with arithmetic averages (UPGMA) and SAHN (Sequential, agglomerative, hierarchical and nested clustering parameters) was constructed using the NTSYSpc 2.11 program (Rohlf, 2000). The polymorphism information content (PIC), major allele frequency, gene diversity (He) and heterozygosity (Ho) were done using Power Marker v3.25 (Liu and Muse, 2005).

Results

1. Summary statistics of 11 morphological quantitative traits and phenotypical genetic diversity analysis of 104 garlic germplasm from China and Egypt

Mean, minimum, maximum, standard deviation and coefficient of variation of traits are shown in Table 3. The CV values of 11 traits exhibited a high variation level (>40%). The range of the mean values showed that the garlic germplasm were significantly different in all traits. The number of cloves per bulb showed the highest degree of variation (CV= 56.27). However, the bulb length showed the lowest coefficient of variation (CV= 15.26).

2. Morphological characterization reveals the phylogenetic relationship of garlic germplasm from China and Egypt

A dendrogram of all 104 garlic germplasms from China and Egypt was constructed based on the morphological traits (Fig. 1). The similarity coefficient among garlic germplasm ranged from 0.40 to 0.95 with an average value of 0.67. By cluster analysis, all garlic germplasm were separated into two main clusters (or groups), I and II, at similarity 0.41. Cluster I included 71 germplasms, 66 garlic germplasms from China and only five germplasms from Egypt. Similarly, cluster II consisted of 33 germplasms, which included 76.19 % of Egyptian garlic germplasm (16 germplasm) and 17 germplasm from China. More specifically, at 0.47 genetic similarity, cluster I was divided into two sub-groups (A and B). Most of garlic germplasm in this cluster (64 accessions, 90.14%) appear in sub-group A, which comprised 63 germplasm from China and only one germplasm from Egypt. However, only 7 garlic

germplasm were presented in sub-group B, which included 4 germplasm from Egypt and 3 germplasm from China. Likewise, cluster II was separated into two sub-groups (C and D) when genetic similarity was 0.47. Out of 33 germplasm presented in cluster II, 10 garlic germplasm were clustered in sub-group C, which comprised nine accessions from China and only one germplasm from Egypt. Out of 23garlic germplasm in sub-group D, 15 germplasm were from Egypt, which counted 71.43% of Egyptian garlic germplasm. It was obvious that there were certain correlation between garlic germplasm origin and their morphological traits although some germplasm from China and Egypt went together.

Table 3: Mean, minimum, maximum, standard deviation (SD) and coefficient of variation (CV) of morphological quantitative traits

Morphological traits	Mean	Min	Max	SD	CV%
Plant height (cm)	46.61	18.80	72.34	11.19	24.01
Plant breadth (cm)	23.77	4.00	61.70	12.96	54.51
Leaf length (cm)	32.82	10.80	63.78	8.94	27.24
Leaf width (cm)	1.59	0.40	2.98	0.61	38.34
Number of leaves per plant	7.11	2.50	10.80	2.08	29.21
Overground pseudostem height (cm)	21.46	2.65	52.66	8.01	37.34
Overground pseudostem diameter (cm)	1.05	0.25	2.16	0.39	36.84
Bulb weight (g)	16.27	0.60	38.20	7.98	49.00
Bulb length (cm)	3.23	1.70	4.24	1.28	15.26
Bulb diameter (cm)	3.07	1.03	4.60	0.68	22.14
Number of cloves per bulb	4.61	1.00	13.00	2.59	56.27

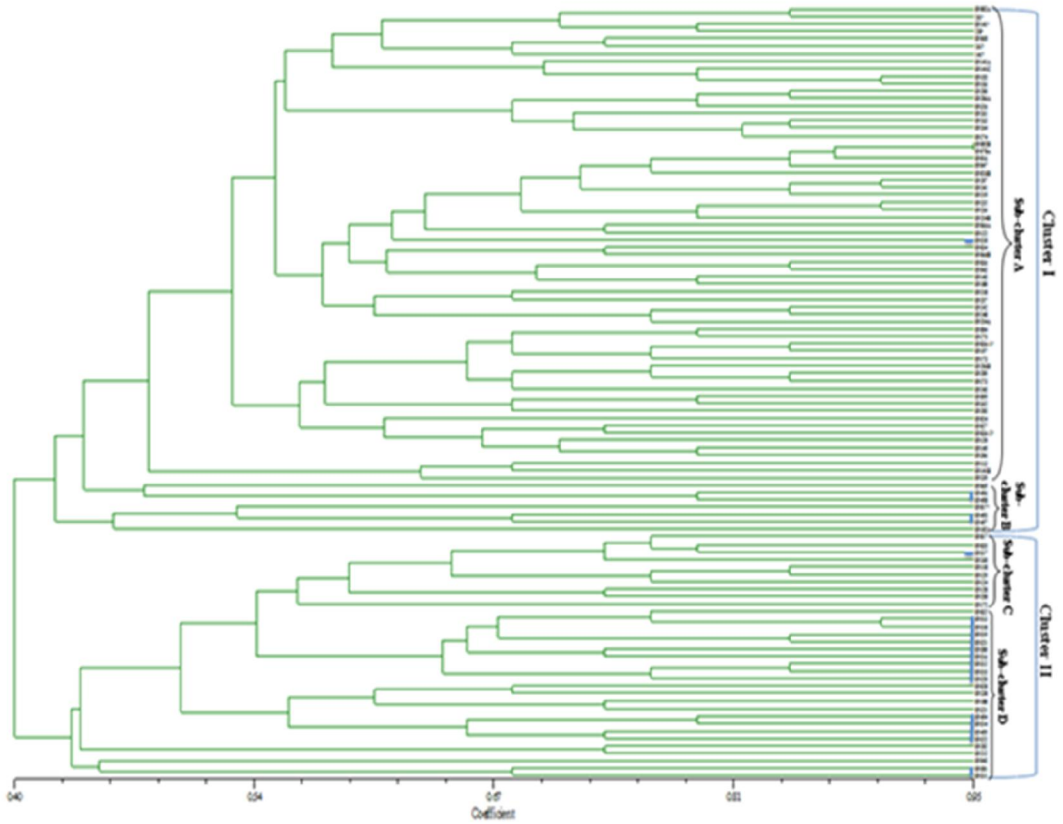

Fig. 1: Cluster dendrogram of 104 garlic germplasm from China and Egypt based on phenotype traits

3. SSR markers development

A total of hundred SSR primers were designed and used to amplify the DNA templates of nine germplasm entries in order to test its accuracy, number of alleles and product size. Primers that failed

to amplify or amplified complicated patterns that were difficult to score were excluded after repeat amplification. Consequently, among them, 16 high-quality microsatellites primer pair listed on Table 4 were used to study the genetic diversity of 104 garlic germplasm from China and Egypt.

A total of 45 alleles were revealed with these 16 SSR primers and the numbers of alleles per locus ranged from 2 to 4 with average polymorphic alleles per locus were 2.81. The fragment size ranged from 76 to 160 bp in Table 4. The maximum number of amplified product (4) was observed in the profiles of the primer G055, G084, G101 and G263. However, the minimum number of amplified product (2) was recorded with primer G003, G129, G250, G293, G307, G335B and G336B. The aver PIC ranged from 0.1463 to 0.6175 with an average 0.3968 (Table 4).

Table 4: SSR primers sequences, repeat motif, product size, number of alleles amplified amomg104 garlic germplasm in this investigation

Primer ID	Forward sequence Reverse sequence	Repeat motif	Tm (°C)	Product size (bp)	Number of alleles	PIC
G003	AGGCATTATTATTTTTCCAGA TCAGTTCAGTTATCAGCTTCC	8 (AC)	55	101-105	2	0.3451
G055	GCAGCAATTTCAATTTCAACA GCAGGAGACTTGTCCACTGTA	6 (GCA)	55	81-100	4	0.4269
G084	ACGTGCGAAGCTAGTGAAGT GAATGAATATCTGGGGCTCA	7 (GA)	55	105-110	4	0.6175
G095	GCCGGCTTACGTAACTTTTA AAGGCCATCTTTCTCCTCTC	6 (GA)	55	112-125	3	0.3946
G101	CCATGCATTCCATTTCACTT AGATCAGCAATGGAGCTTGT	3 (TCT)	55	76-85	4	0.5723
G129	GAATTCGCTAATTTGGCAGT CGAATCTCCTTCCAACGTAA	2 (GA)	55	84-90	2	0.3748
G137	CGAAGAAGATGGCAAGTTTT CCGTTGTATCCCTTCCTTTT	3 (GCT)	55	79-85	3	0.1463
G250	CACCACCACTTGGTACATAAA TGAATAACATAGAGGGGGACA	7 (CT)	55	134-140	2	0.3735
G258	TGATGGATGACTACAATAGTTCG TCCATTCTGGGTTTGTCTCT	5 (GAG)	55	160-170	3	0.4848
G263	AAAAACCAGAATAATACCTCAACG CAAACTCCTTTTGGCAATTA	6 (TC)	55	85-95	4	0.5883
G293	AGAGACAACGAAGCCATCTG GCGAAGCAATGGAAGTTCT	5 (TGC)	55	82-90	2	0.3374
G307	GAGCATTTCTCCATTTGACG AAGCCTATACATACCAGCATAACA	6 (GAT)	55	85-90	2	0.3735
G335B	TACGCTGCTGTTCAAATTCA CATCAACAACGAGAACGAGA	5 (TCG)	55	85-90	2	0.1833
G336A	GGGTGCAGAGCTATTTAATCC TAATCTTCACCAACCCCATC	7 (TGG)	55	83-95	3	0.3838
G336B	TACGCTGCTGTTCAAATTCA CATCAACAACGAGAACGAGA	5 (TCG)	55	85-90	2	0.1891
G372	CCCACATATCCCACTACCAA GGAGGAATCTAGGCCCACTA	6 (TA)	55	111-120	3	0.5578

4. Statistical analysis of genetic diversity among Chinese, Egyptian, and all germplasm from China and Egypt using SSR markers

Various genetic diversity parameters, major allele frequency, gene diversity and heterozygosity, were calculated to compare among Chinese, Egyptian, and all garlic germplasm (Table 5). The major allele frequency for each SSR locus ranged from 0.4578 to 0.9639 for Chinese garlic with an average 0.6438, 0.3810 to 0.8333 for Egyptian garlic with an average 0.5745 and for all germplasm ranged from 0.4183 to 0.9183 with an average 0.6199. However, the average genotype diversity was 0.4495 (ranged from 0.0703 to 0.6772), 0.5133 (ranged from 0.2891 to 0.6621) and 0.4719 (ranged from 0.1529 to

0.6670) for Chinese, Egyptian and all garlic germplasm, respectively. Moreover, the heterozygosity value for SSR locus ranged from 0.0000 to 0.9639 (mean 0.4992) for Chinese garlic. In addition, the heterozygosity value for Egyptian garlic germplasm ranged from 0.0000 to 0.9524 with an average 0.4751. However, the recorded average of heterozygosity for all garlic germplasm was 0.4945, which ranged from 0.0000 to 0.9231 (Table 5).

Table 5: Major allele frequency, gene diversity and heterozygosity for used SSR primers among Chinese, Egyptian and all garlic germplasm.

Primer ID	Major allele frequency			Gene diversity			Heterozygosity		
	Chinese	Egyptian	All germplasm	Chinese	Egyptian	All germplasm	Chinese	Egyptian	All germplasm
G003	0.6687	0.6667	0.6683	0.4431	0.4444	0.4434	0.5663	0.6667	0.5865
G055	0.6325	0.4524	0.5865	0.4816	0.6111	0.5206	0.6627	0.9048	0.7115
G084	0.4819	0.5238	0.4904	0.6772	0.5839	0.6670	0.0120	0.0476	0.0192
G095	0.6988	0.8333	0.7260	0.4663	0.2891	0.4351	0.2651	0.2381	0.2596
G101	0.4639	0.5000	0.4327	0.6408	0.6179	0.6453	0.9157	0.9524	0.9231
G129	0.5241	0.5250	0.5146	0.4988	0.4988	0.4996	0.9518	0.6500	0.8932
G137	0.9639	0.7381	0.9183	0.0703	0.3980	0.1529	0.0241	0.0476	0.0288
G250	0.5602	0.5476	0.5385	0.4927	0.4955	0.4970	0.3976	0.5238	0.4231
G258	0.6265	0.4286	0.5769	0.5214	0.6349	0.5592	0.5301	0.4762	0.5192
G263	0.4578	0.3810	0.4183	0.6462	0.6587	0.6593	0.9157	0.7619	0.8846
G293	0.6988	0.6429	0.6875	0.4210	0.4592	0.4297	0.6024	0.2381	0.5288
G307	0.5181	0.6190	0.5385	0.4993	0.4717	0.4970	0.9639	0.7619	0.9231
G335B	0.9398	0.6667	0.8846	0.1132	0.4444	0.2041	0.0000	0.0000	0.0000
G336A	0.6205	0.5952	0.5673	0.4710	0.5159	0.4991	0.6386	0.6190	0.6346
G336B	0.9277	0.6905	0.8798	0.1341	0.4274	0.2115	0.0000	0.0476	0.0096
G372	0.5181	0.3810	0.4904	0.6148	0.6621	0.6289	0.5422	0.6667	0.5673

5. Phylogenetic relationship of 104 garlic germplasm from China and Egypt based on 16 SSR markers

Using NTSYS-pc2.11 software, a dendrogram was constructed according to 16 SSR markers of 104 garlic germplasm. By UPGMA analysis, the average genetic similarity coefficient of 0.77 and range from 0.54 to 1.00 were gotten. The genetic similarity data obtained from SSR data were used to investigate the difference among garlic germplasm from Egypt and China at the DNA level (Fig. 2). All garlic germplasm were clustered into three main groups (I, II and III) when genetic similarity was 0.63. Most of garlic germplasm (64 germplasm) presented in cluster I, which were divided into two sub-groups (A and B) at genetic similarity of 0.82. Sub-group A contained 50 garlic germplasm, the majority of which (45 germplasm) came from China and five germplasm from Egypt. Meanwhile, of 14 garlic germplasm, eight garlic germplasm from Egypt and six germplasm from China were grouped in sub-group B. Cluster II contained 28 germplasm, in which only one germplasm was from Egypt and the rest were from China. Cluster III consisted of seven germplasm from Egypt and only five-garlic germplasm from China. The dendrogram revealed that there is also certain correlation between the genetic relationships and origin of the germplasm. In general, most of Egyptian garlic germplasm (15 germplasm of 21, 71.43%) were clustered in sub-group B in cluster I (8 germplasm) and cluster III (7 germplasm). The genetic relationships among accessions are basically similar in two dendrograms.

6. The Phylogenetic relationship of 104 garlic germplasm from China and Egypt based on the integration of morphological and SSR markers

Based on similarity coefficient values, the unweighted pair group method arithmetic average (UPGMA) cluster was constructed to observe the genetic relatedness among garlic germplasm from China and Egypt (Fig. 3). The value of similarity coefficient ranged from 0.0 to 0.25 and all the 104 germplasm were divided into two major clusters. The cluster II at similarity 0.23 was divided into two sub-clusters (A and B). Egyptian garlic germplasm were grouped together in both cluster. Out of 21 Egyptian garlic germplasm, 11 accessions were placed in sub-cluster B (Cluster I), nine accessions were grouped in cluster II and only one accessions was found in sub-cluster A (cluster I). The results of the integrated cluster (phenotype traits and SSR data) reveled that most of Egyptian garlic germplasm were grouped pursuant to their geographic origin. Where, 54.4 % of Egyptian accessions were grouped in sub-cluster B and 42.86 % were placed in cluster II.

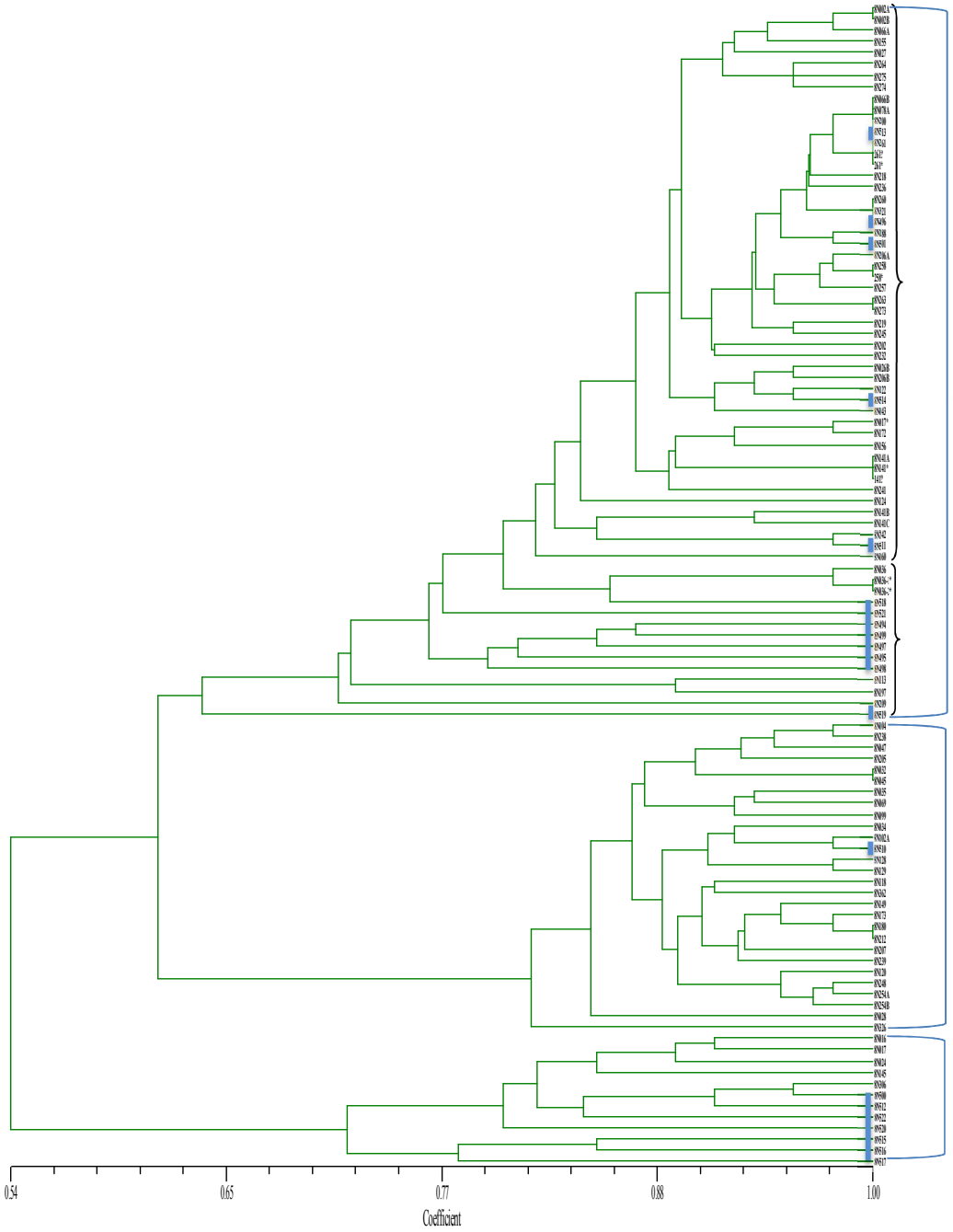

Fig. 2: Dendrogram of 104 garlic germplasm from China and Egypt based on the UPGMA method and 16 new SSR primers.

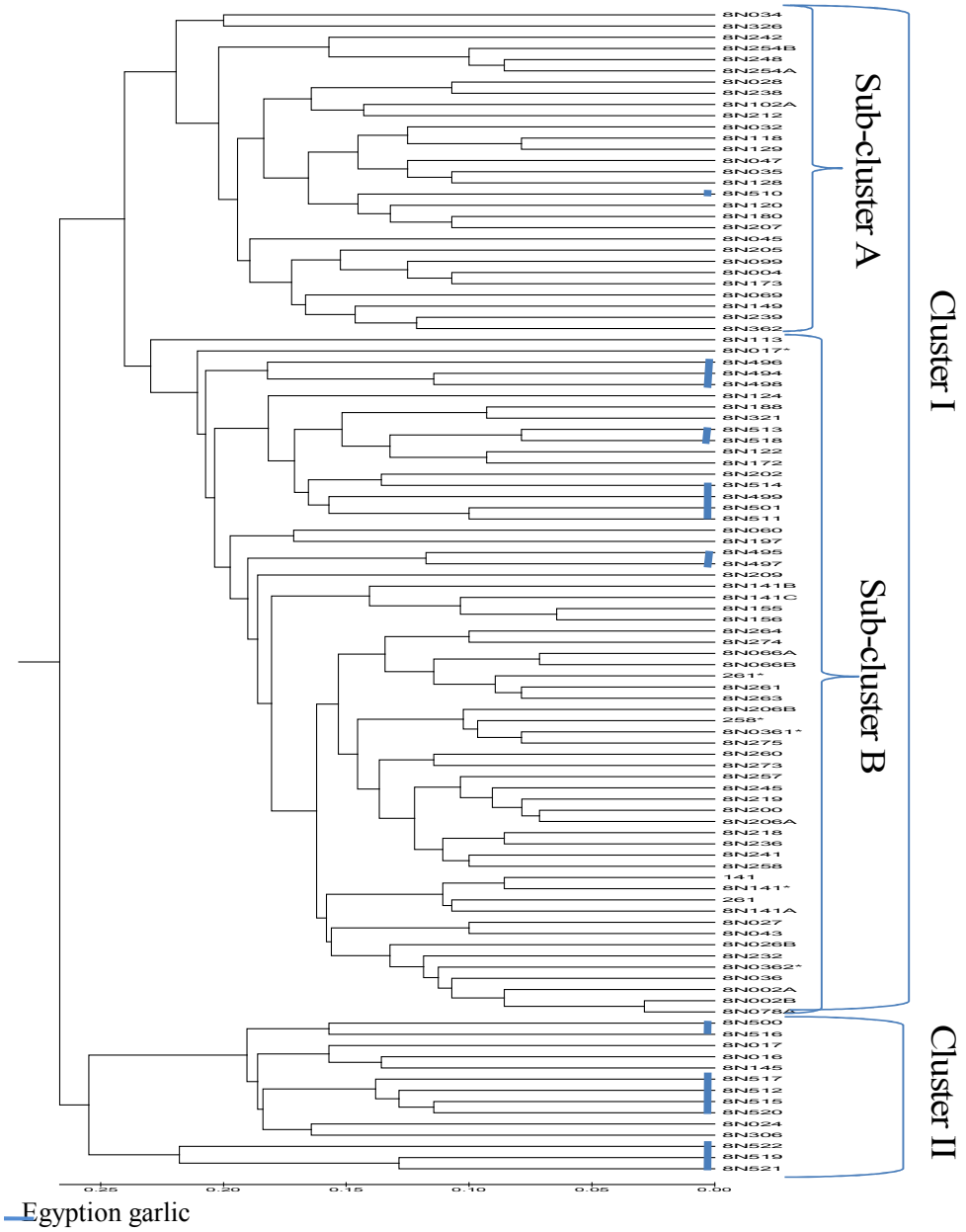

Fig. 3: Cluster dendrogram of 104-garlic germplasm from China and Egypt based on the UPGMA method, phenotype traits and 16 new SSR primers.

7. Effects of genetic improvements through clonal selection

Cluster analysis based on morphological traits (Fig. 1) showed that parent and its offspring were grouped into different groups (*e.g.,* clone offspring 8N017* appeared in sub-group B and its parent 8N017 in sub-group C). Moreover, clone offspring 8N258* selected and its parent 8N258 were divided into different sub-group, A and C, respectively. Likewise, the clone 261º by bulk selection, its parent 8N261and its sister clone by single selection (261*) were grouped in different nodes of sub-group A. On the other hand, the dendrogram based on SSR data by 16 primers showed different results in some circumstances (Fig. 2). The offspring clones 8N017* presented in cluster I, sub-group A, whiles its parent 8N017 in cluster III (Fig. 2). In fact, out of 16 novel primers, eight primers could reveal the genetic differences between selected clone offspring 8N017* and its parent 8N017 (Fig. 4).

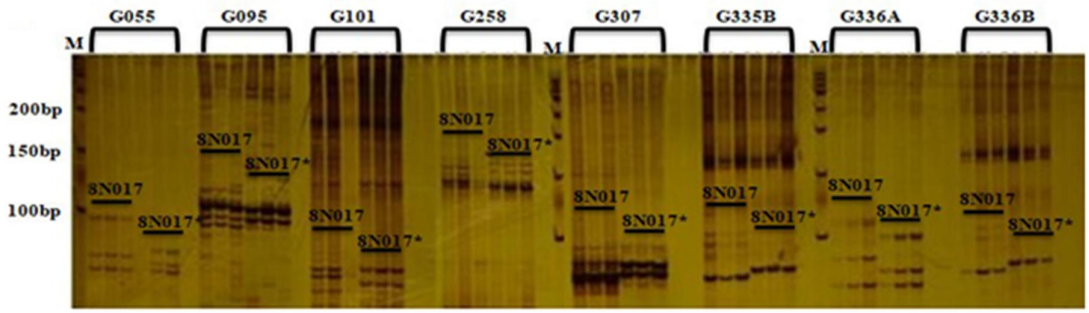

Fig. 4: Genetic difference between selected clone offspring 8N017* and its parent 8N017

Discussion

The estimation of degree of genetic variation among garlic germplasm both in morphological and molecular level is the essential study, which may provide a general guide for germplasm selection for breeding purposes. In addition, it is necessary to identify true biodiversity to prevent redundancies and thus space and preservation costs in germplasm bank. Consequently, in this study, we investigated the morphological and molecular genetic diversity of garlic germplasm to clarify the genetic diversity status of all accession and two countries and to point out Egyptian garlic accession could contribute their genetic diversity to the world garlic gene pool.

Morphological characterization is the first step in the description and classification of germplasm (Smith and Smith, 1989). Moreover, phenotypic traits measurement is considered as an important and fundamental method for diversity estimation (Zhang *et al.*, 2018). In the development of breeding populations, plant breeders can use genetic similarity information to complement phenotypic information (Greene *et al.*, 2004). Classification of garlic germplasm based on morphological traits showed great variation among tested germplasm. Garlic germplasm from the same geographical regions clustered together. Similarities observed in several studies of Baghalian *et al.* (2006), Zahedi *et al.* (2007) and Egea *et al.* (2017). On the other hand, some garlic accessions based on their morphological traits were not grouped in the same cluster. This might be due the reasons of garlic germplasm exchange between countries or commercial trade and or farmers renamed the garlic variety from the same region (da Cunha *et al.*, 2014).

Compared with phenotypic assessment, molecular marker technology is an efficient supplement and alternative to phenotypic measurements (Zhang *et al.*, 2018). It has become an essential tool to identify and manage genetic diversity. In current investigation, a total of 104-garlic germplasm from China and Egypt were classified using 16 new SSR primers. The clustering with SSR data indicated that there were obvious association between clusters and original location within tested germplasm. This is in agreements with the study by Jo *et al.* (2012) who revealed that genetic relationship is correlated with geographical region among garlic germplasm collected from five different countries. Similarly, Ma *et al.* (2009) showed that the major groups were with moderate relation with geographical location by cluster analysis. On the other hand, our result is in contrast with Ipek *et al.* (2003, 2008); Volk *et al.* (2004); Baghalian *et al.* (2005, 2006); Mario *et al.* (2008); Asili *et al.* (2010) and Jabbes *et al.* (2012), who reported that there is no relationship between ecological conditions and genetic divergence. This may be related to the amount and origin of the tested germplasm, and also the distinguishability of the chosen characters and primers. The obtained results based on morphological cluster and SSR cluster revealed that revealed that these two clusters are relatively congruence with each other and mentioned the relationship between clusters and geographical region.

In present investigation, a total of 45 alleles were detected with 16 new SSR primers and the average allele number per locus was ranged from 2 to 4 alleles with an average 2.81. The average polymorphic alleles per locus were high variable in different analyses. For instance, Lee *et al.* (2011), using 50 SSRs loci on 20 germplasm (five Allium species), reported that the average number of amplified alleles ranged from 1.452 to 1.910 per locus. Nevertheless, Ma *et al.* (2009) reported an average of 8 alleles per locus on 90 garlic germplasm. Also, Zhao *et al.* (2011) concluded that average allelic richness was 14.1 alleles per locus for eight SSR loci on 613 germplasm of garlic. Jo *et al.* (2012), using seven simple sequences revealed an average number of 5.285 alleles per locus on 120 garlic

germplasm. Furthermore, an average of 4.4 alleles per loci was identified for 16 SSR loci (Cunha *et al.*, 2012). It seems to us that the average allele number per locus depends on plant species, the degree of diversity within germplasm or germplasm purity and types of motifs in SSRs used.

Furthermore, the cluster dendrogram according to phenotype and molecular data confirmed relatively genetic relationship with geographical region. This might be confirmed the importance of analyzed the molecular and morphological data together to ensure a reliable identification of duplicates and core collection in line with specific interests of genetic breeding programs (da Cunha *et al.*, 2014).

For overall identification of genetic diversity in a crop, it would be better to use the more SSR primers distributed on the whole genome. The 16 SSR primers should be the newly additional effective tools for garlic even Allium species to identify genetic diversity, confirm clone selection and select promising materials.

Conclusion

In this study, we developed 16 novel microsatellites primers, which could be efficient for the evaluation of garlic genetic diversity. There was high genetic diversity in Chinese and Egyptian garlic germplasm based on morphological and molecular markers. Garlic germplasm were classified into two main clusters based on morphological traits and three main groups based on SSR analyses. In both clustering analysis, based on phenotypic and SSR data, there were relationship between clusters and original location. Our study might be helpful to identify different germplasm based on obtained rich information for breeding new cultivars of garlic in the future.

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Reference

Agrawal, M.K., M.S. Fageria, and R.S. Dhaka, 2003. Garlic breeding-A Review. Agric. Rev. 24:70 – 74.

Al-Zahim M, H J Newbury, B V. Ford-Lioyd 1997. Classification of genetic variation in garlic (*Allium sativum* L.) revealed by RAPD. Hort. Science, 32:1102-1104.

Asili, A., J. Behravan, M.R. Naghavi, and J. Asili, 2010. Genetic diversity of Persian shallot (*Allium hirtifolium*) ecotypes based on morphological traits, allicin content and RAPD markers. J. Medicinal and Aromatic Plants, 1:1-6.

Baghalian, K., M.R. Naghavi, S.A. Ziai, and H.N. Badi, 2006. Post-planting evaluation of morphological characters and allicin content in Iranian garlic (*Allium sativum* L.) ecotypes. Scientia Horticulturae, 107:405-410.

Baghalian, K., S.A. Ziai, M.R. Naghavi, B.H.A Naghdi, and A. Khalighi, 2005. Evaluation of allicin content and botanical traits in Iranian garlic (*Allium sativum* L.) ecotypes. Scientia Horticulturae, 103:155–166.

Cunha, C.P., E.S.S. Hoogerhride, M.I. Zucchi, M. Monteiro, and J.B. Pinheiro, 2012. New microsatellite markers for garlic, *Allium sativum* (Alliaceae). American Journal of Botany: e17-e19. https://doi: 10.3732/ajb.1100278.

Da-Cunha, C.P., F.V. Resende, M.I. Zucchi, and J.B. Pinheiro, 2014. SSR-based genetic diversity and structure of garlic accessions from Brazil. Genetica 142: 419-431. https://doi.org/10.1007/s10709-014-9786-1.

Egea, L.A., R. Mérida-García, A. Kilian, P. Hernandez, and G. Dorado, 2017. Assessment of Genetic Diversity and Structure of Large Garlic (*Allium sativum*) Germplasm Bank, by Diversity Arrays Technology "Genotyping-by-Sequencing" Platform (DArTseq). Front. Genet. 8:98. https://doi: 10.3389/fgene.2017.00098

Emanuelli, F., S. Lorenzi, L. Grzeskowiak, V. Catalano, M. Stefanini, M. Troggio, S. Myles, J.M. Martinez-Zapater, E. Zyprian, F.M. Moreira, and M.S. Grando, 2013. Genetic diversity and population structure assessed by SSR and SNP markers in a large germplasm collection of grape. BMC Plant Biology, 13: 39

Food and Agricultural Organization of the United Nations, 2012. Economic and Social Department: The Statistical Division.

Figliuolo, G., V. Candido, G. Logozzo, V. Miccolis, and P.L. Spagnoletti-Zeuli, 2001. Genetic evaluation of cultivated garlic germplasm (*Allium sativum* L. and *A. ampeloprasum* L.). Euphytica 121:325-334.

Frank, M.Y., H. Naxin , Q.G. Yong , L. Ming-Cheng , M. Yaqin , H. Dave , R. Gerard, J.D. Lazo, and D. Olin, 2008. BatchPrimer3: A high throughput web application for PCR and sequencing primer design. BMC Bioinformatics, 9: 253

Fritsch, R.M., and N. Friesen, 2002. Evolution, Domestication and Taxonomy. In: Allium Crop Sciences: Recent Advances. Rabinowitch HD, & Currah L, Eds., pp. 5-30, CAB International, ISBN 0-35199-510-1, Wallingford, U. K.

Greene, S.L., M. Gritsenko, and G. Vandemark, 2004. Relating morphologic and RAPD marker variation to collection site environment in wild populations of red clover (*Trifolium pratense* L.). Genet. Resour. Crop Evol., 51:643–653.

Gvozdanovic, V.J., M. Vasic, J. Cervenski, and D. Bugarski, 2004. Genotype and environment effects on yield and quality of winter garlic. Genetica 36:161-170.

Ipek, M., A. Ipek, and P.W. Simon, 2003. Comparison of AFLPs, RAPD markers, and isozymes for diversity assessment of garlic and detection of putative duplicates in germplasm collection. J. Am. Soc. Hort. Sci. 128:246–252.

Ipek, M., A.I. Philipp, and P.W. Simon, 2008. Molecular characterization of Kastamonu garlic: an economically important garlic clone in Turkey. Scientia Horticulturae, 115:203–208.

IPGRI ECP/GR, and AVRDC, 2001. Descriptor for Allium (Allium ssp.). International Plant Genetic Institute, Rome, Italy; European Cooperatine Programme for Crop Resources Netuorks (ECP/GR), Asian Vegetable Research and Development Center, Taiwan.

Jabbes, N., I. Arnault, J. Auger, B.A.M. Dridi, C. Hannachi, 2012. Agro-morphological markers and organo-sulphur compounds to assess diversity in Tunisian garlic landraces. Scientia Horticulturae 148:47-54.

Jo, M.H., I.K. Ham, K.T. Moe, S.W. Kwon, F.H. Lu, Y.J. Park, W.S. Kim, M.K. Won, T.I. Kim, and E.M. Lee, 2012. Classification of genetic variation in garlic (*Allium sativum* L) using SSR markers. Australian Journal of Crop Science, 6:625-631.

Karuri, H.W., E.M. Ateka, R. Amata, A.B. Nyende, A.W.T. Muigai, E. Mwasame, and S.T. Gichuki, 2010. Evaluating diversity among Kenyan sweet potato genotypes using morphological and SSR markers. Int. J. Agric. Biol., 12:33–38

Lammerink, J., and A.R. Wallace, 1987. Effect of clonal selection and planted clove weight on yield of garlic. In: Proceeding of Annual Conference. Agronomy Society of Newzeland, 17:7–10.

Lampasona, G.S., L. Martinez, and J.L. Burba, 2003. Genetic diversity among selected Argentinean garli clones (*Allium sativum* L) using AFLP (Amplified Fragment Length Polymorphism). Euphytica 132:115-119.

Latif, M.A., M.M. Rahman, M.E. Ali, S. Ashkani, and M.Y. Rafii, 2013. Inheritance studies of SSR and ISSR molecular markers and phylogenetic relationship of rice genotypes resistant to tungro virus. Comptes Rendus Biologies 336: 125-133.

Lee, G.A., S.J. Kwon, Y.J. Park, M.C. Lee, H.H. Kim, J.S. Lee, S.Y. Lee, J.G. Gwag, C.K. Kim, and K.H. Ma, 2011. Cross-amplification of SSR markers developed from *Allium sativum* to other Allium species. Scientia Horticulturae, 128:401-407.

Liu, K., and S.V. Muse, 2005. PowerMarker: an integrated analysis environment for genetic marker analysis. Bioinformatics, 21: 2128-2129.

Ma, K.H., J.G. Kwag, W. Zhao, A. Dixit, G.A. Lee, H.H. Kim, I.M. Chung, N.S. Kim, J.S. Lee, J.J. Ji, T.S. Kim, and Y.J. Park 2009. Isolation and characteristics of eight novel polymorphic microsatellite loci from the genome of garlic (*Allium sativum* L.). Scientia Horticulturae 122:355-361.

Mario, P.C., B.V. Viviana, and I.A. Marı´a, 2008. Low genetic diversity among garlic accessions detected using RAPD. Chilean J. Agric. Res. 68:3–12.

Moncrief, I., C. Garzon, S. Marek, J. Stack, A. Gamliel, P. Garrido, F. Proano, M Gard, H. Dehne, and J. Fletcher, 2016. Development of simple sequence repeat (SSR) markers for discrimination among isolates of *Fusarium proliferatum*. Journal of Microbiological Methods. 126: 12-17

Murray, J.M., and W. Thompson, 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 8:4321-4325.

Pandian, S., L. Satish, R. Rameshkumar, P. Muthuramalingam, A.S. Rency, P. Rathinapriya, and M. Ramesh, 2018. Analysis of population structure and genetic diversity in an exotic germplasm collection of *Eleusine coracana* (L.) Gaertn. Using genic-SSR markers. Gene 653: 80-90.

Rakoczy-Trojanowska, M., and H. Bolibok, 2004. Characteristics and a comparison of three classes of microsatellite-based markers and their application in plants. Cel. Mol. Bio. Let. 9:221–238.

Rohlf, F.J. 2000. NTSYS-$_{PC}$: Numerical Taxonomy and Multivariate Analysis System. Version 2.11x. Exeter Software, Setauket, New York, USA, P. 83.

Singh, B., N. Singh, S. Mishra, K. Tripathi, B.P. Singh, V. Rai, A.K. Singh, and N.K. Singh, 2018. Morphological and Molecular Data Reveal Three Distinct Populations of Indian Wild Rice Oryza rufipogon Griff. Species Complex. Front. Plant Sci. 9:123. https://doi: 10.3389/fpls.2018.00123

Smith, J.S.C., and O.S. Smith, 1989. The description and assessment of distances between inbred lines of maize: the utility of morphological, biochemical and genetic descriptors and a scheme for the testing of distinctiveness between inbred lines. Maydica, 34:151–161.

Sokal, R.R., and C.D. Michener, 1958. A statistical method for evaluating systematic relationships: Univ. Kansas Sci. Bull. 38:1409-1438.

Vijayanand, V., N. Senthil, S. Vellaikumar, and M. Paramathma, 2009. Genetic diversity of Indian jatropha species as revealed by morphological and ISSR marker. J. Crop Sci. Biotech. 12:115-120.

Volk, G.M., A.D. Henk, and C.M. Richards, 2004. Genetic diversity among U.S. garlic clones as detected using AFLP methods. J. Am. Soc. Hort. Sci. 129:559–569.

Zahedi, B., A.K. Kasha, Z. Zamani, G.H. Mosahebi, and M. Hassani, 2007. Evaluation of Iranian garlic (*Allium sativum* L.) genotypes using multivariate analysis methods based on morphological characteristics. Biotechnology 6:353-356.

Zhang, Z., W. Xie, J.Z.X. Zhao, Y. Zhao, and Y. Wang 2018. Phenotype and SSR-based estimates of genetic variation between and within two important *Elymus* species in western and northern China. Genes 9: 147, https://doi:10.3390/genes9030147.

Zhao, W.G., J.W. Chung, G.A. Lee, K.H. Ma, H.H. Kim, K.T. Kim, I.M. Chung, J.K. Lee, N.S. Kim, S.M. Kim, and Y.J. Park, 2011. Molecular genetic diversity and population structure of a selected core set in garlic and its relatives using novel SSR markers. Plant Breeding, 130:46-54.